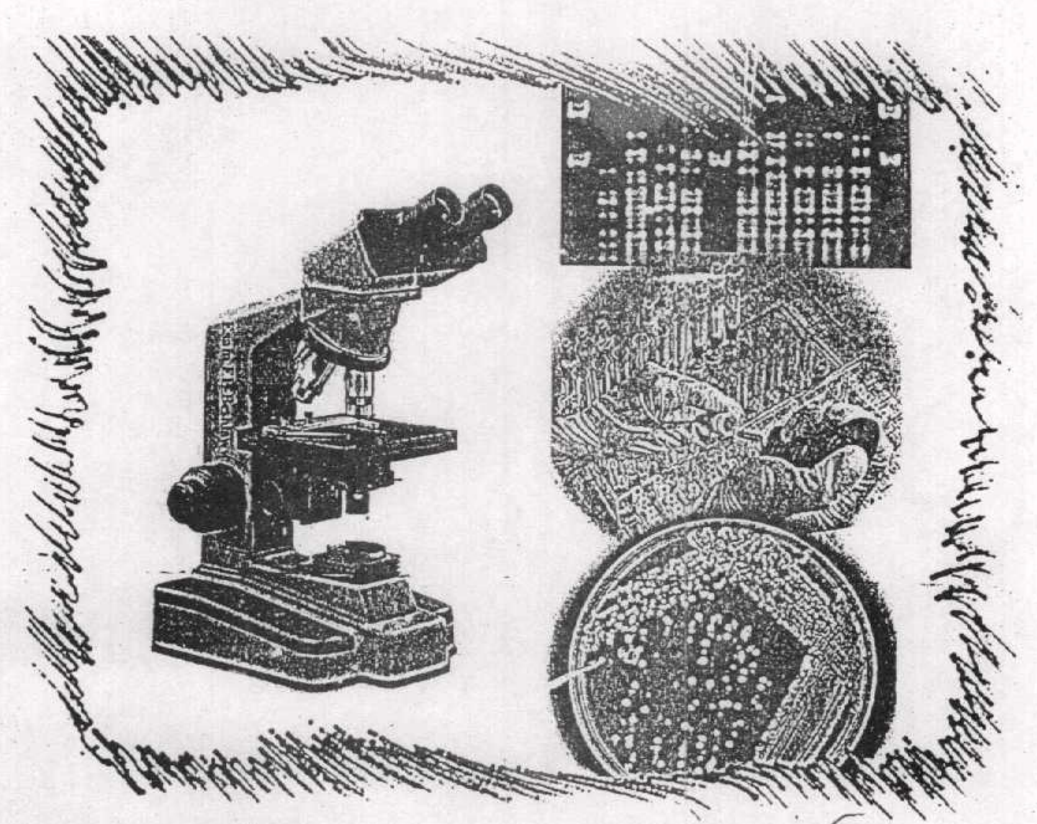
Practical Microbiology

Part II



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Specimen Collection and Handling

Ideally, tissue would be collected separately at the time of autopsy and would include tissue from each organ and from several areas of each organ. Fixed tissue from multiple organs can be combined in one container.

If specimens are limited, priority should be given to providing fixed tissue, especially from the primary affected organ (for example, brain tissue for a meningoencephalitis case). If a specifically affected area is noted by gross pathology or preliminary histopathology, it is helpful to submit tissue from this area.

Fixed tissue

- Fixed tissue should either be in 10% buffered formalin or paraffin-embedded
- Fixed tissue is used for routine H&E stains and special stains as well as immunohistochemistry and in situ hybridazation.
- If fixed tissue is submitted to CDC more than 2 weeks after it is collected, paraffin-embedded tissue is preferred for submission.
- Over-fixation will form a strong formalin bond in tissue and make antigen retrieval more difficult.
- Blocks rather than slides are also preferred since epitopes and nucleic acids can degenerate more quickly once tissue is prepared for slides thus decreasing sensitivity of special assays.
- Fixed tissue from multiple organs can be combined in one container.
- Fixed tissue should be stored and shipped at room temperature.
  Do not freeze fixed tissue.

Fresh frozen tissue

- Frozen tissue is used for culture and molecular techniques including specific primer and consensus polymerase chain reaction (PCR).
- Specimens should be collected aseptically and as soon as possible after death.
- A separate sterile instrument should be used for each collection site and each specimen should be placed in a separate sterile container in small amounts of viral transport media or saline.
- Frozen tissue should be stored at -70° C and shipped on dry ice.

Body fluids

- 5-10 cc of cerebrospinal fluid and 5 cc of heart blood (in a marbled red tube top) collected postmortem may also be used for testing.
- Body fluid specimens should be kept refrigerated and shipped with a cold pack. If a respiratory infection is suspected, a postmortem nasopharyngeal swab can be collected in a sterile container with viral transport media for viral culture.

Infectious Disease

The proper collection of a specimen for culture is the most important step in the recovery of pathogenic organisms responsible for infectious disease. A poorly collected specimen may lead to failure in isolating the causative organism(s) and/or result in the recovery of contaminating organisms.

Basic Concepts for Specimen Collection

- Collect the specimen from the actual site of infection, avoiding contamination from adjacent tissues or secretions.
- Collect the specimen at optimal times (for example, early morning sputum for AFB culture).
- Collect a sufficient quantity of material. Use appropriate collection devices: sterile, leak-proof specimen containers. Use appropriate transport media (anaerobe transport vials, Amies or Stuart's for bacterial culture, Cary-Blair for stool culture, M4 for viral, Chlamydia, and ureaplasma cultures).
- Whenever possible, collect specimens prior to administration of antimicrobials.
- Properly label the specimen and complete the test request form. The source of specimen is required.
- Minimize transport time. Maintain an appropriate environment between collection of specimens and delivery to the laboratory.
- If appropriate, decontaminate the skin surface. Use 70- 95% alcohol (ALC) and 1-2% tincture of iodine (TOI) to prepare the site. Allow a contact time of two minutes to maximize the antiseptic effect.
- Package each specimen separately in its own sealable transport bag.
- Isolates submitted on agar plates or as mixed cultures are unacceptable.
- Slides must be labeled individually on the side the sample is inoculated.

Abscess

- Decontaminate the surface with 70-95% ALC and 1-2% TOI.
- Collect purulent material aseptically from an undrained abscess using a sterile needle and syringe. Open miliary abscesses with a sterile scalpel and collect the expressed material with a sterile needle and syringe.
- Expel air from the syringe, remove the needle, and cap the syringe. Alternatively, transfer 5-10 mL of theaspirated material to an anaerobic transport vial. Transport immediately.
- Swabs are a poor choice because they dry easily and there is limited amount of material obtained. Swabs are not acceptable for mycobacterial or fungal cultures.

Blood (bacteria only)

- Gather the collection blood bottles or tubes needed.
- Swab the tops of each blood culture bottle and/or the stopper of an Isolator® or SPS tube with alcohol. Do not allow alcohol to pool, as it could enter the system and kill organisms. Allow to dry while preparing the patient.
- Cleanse the skin with 70-95% ALC.
- Cleanse the skin with 1-2% TOI. Move in an everincreasing circular pattern, starting at the point of projected needle insertion.
- Apply a tourniquet proximal to the point of venous entry. The venipuncture site should not be palpated following disinfection unless sterile gloves are worn.
- Use a sterile needle and syringe or closed system blood collection tubing. For fastidious microorganisms, use the Vacutainer® system for Isolator® tubes.

- Collect blood. The volume of blood collected is critical. Inoculate the bottles or tubes without changing needles.
    - For adult bacterial culture, inoculate aerobic bottle with 10 mL of blood or the anaerobic bottle with 7 mL of blood. If less than 17 mL is collected for two bottles, inoculate the aerobic bottle with 10 mL and inoculate the anaerobic bottle with the remainder.
    - For pediatric specimens, inoculate 1-3 mL blood into a pediatric bottle.
    - For fungal and AFB cultures, inoculate 5 mL blood into a Bactec™ Myco/F Lytic bottle.
    - The adult Isolator® tubes will accommodate 9.5-10 mL blood. Allow the vacuum to draw in the proper amount of blood. Do not force the blood into the tube.
    - The SPS vacutainers hold 8 mL or 3 mL.
- Invert tubes several times after specimen collection.
- Remove the iodine from the skin after collection of the specimen.
- Label and transport specimens immediately. Do not refrigerate. Hold at room temperature or at 35°C. Note: Isolator® tubes should be used only for isolation of histoplasma and unusual fastidious or intracellular bacteria such as Brucella sp., Francisella sp., Bartonella sp., etc.

Body Fluids, Sterile (except urine and CSF)

- Prepare the skin as for blood cultures.
- Collect the fluid using a sterile needle and syringe.
- Submit 10 mL of the specimen for analysis. Transport the specimen in a capped syringe, or
    - For aerobic and anaerobic organisms, use an anaerobic transport vial to ensure the survival of anaerobic organisms.
    - 10 mL of peritoneal fluid may be added to a blood culture bottle. Peritoneal fluid is the only body fluid that may be cultured in blood culture media.
    - For viral isolation, send 3 mL or less fluid in viral transport medium or a sterile vial.
    - If tuberculosis or fungal infections are suspected, larger volumes are required. Collect in sterile container.
- Immediately transport fungal specimens at 2-8°C, viral specimens at 2-8°C, and all other specimens at ambient temperature.

Bone Marrow

- Physicians should wear gowns, masks, and gloves during specimen collection.
- Prepare skin as for blood cultures.
- Drape the surrounding skin with sterile linen.
- Aspirate the marrow percutaneously using a sterile needle and syringe.
- Transfer 3-5 mL to a sterile tube containing SPS for bacterial AFB and fungal cultures. EDTA is required for viral cultures and molecular tests.
- Transport specimens immediately at ambient temperature.

Cutaneous (fungus only)

- Hair
    - Scrape the scalp with a blunt scalpel.
    - Place specimen in a dry sterile container.
    - Transport at ambient temperature.
    - The following specimens are also acceptable:
        - Hair stubs

- Contents of plugged follicles
- Skin scales
- Hair plucked from the scalp with for :eps

- Nails
  - Cleanse the nail with 70-95% ALC.
  - Remove the outermost layer by scraping with a scalpel.
  - Place specimen in a dry, sterile container.
  - Transport at ambient temperature.
  - The following specimens are also acceptable:
    - Clippings from any discolored or brittle parts of nail
    - Deeper scrapings and debris under the edges of the nail
- Skin
  - Cleanse the skin with 70-95% ALC.
  - Collect epidermal scales with a scalpel, at the active border of the lesion.
  - Place specimen in a dry, sterile container.
  - Transport at ambient temperature.

Ear

- External ear cultures are processed as superficial wounds.
- Middle ear fluid will be processed as a sterile body fluid. If the diagnosis is otitis media, the specimen of choice is middle ear fluid collected by tympanocentesis.

Eye

- Cleanse the skin around the eye with a mild antiseptic.
- Purulent conjunctivitis
  - Collect purulent material with a regular cotton swab.
  - Place the swab into transport media and transport at ambient temperature or 2-8°C for viral cultures.
- Corneal infections
  - Swab the conjunctiva as described above.
  - Collect multiple corneal scrapings and inoculate directly onto bacterial agar media (chocolate agar, potato dextrose agar, and sheep blood agar) or viral transport media.
  - Transport at ambient temperature or 2-8°C for viral cultures.
- Intraocular fluid
  - Collect fluid by surgical needle aspiration.
  - Transport bacterial cultures at ambient temperature, viral cultures at 2-8°C, or frozen for molecular tests.

Nasopharyngeal Swab

- Seat the patient comfortably and tilt the head back.
- Insert a nasal speculum.
- Insert a nasopharyngeal swab (on a malleable wire) through the speculum into the nasopharyngeal area.
- Rotate the swab gently and allow to remain for 20-30 seconds.

- Remove the swab and place in a nongrowthpromoting transport medium (such as the culturette container from which the original swab has been removed). Place swab in M4 media for viral cultures.
- Transport at ambient temperature or 2-8°C for viral cultures.

Prostate

- Cleanse the glans with soap and water.
- Obtain prostate fluid by digital massage through the rectum.
- Collect fluid using a sterile swab.
- Transport at room temperature.
- Alternatively, a urine specimen obtained immediately before and after massage may be submitted for culture.

Sputum

- Assure patient cooperation to get an adequate specimen. ARUP will determine the number of squamous epithelial cells present for specimen adequacy.
- Instruct the patient as follows:
    - Rinse mouth with tap water to remove food particles and debris.
    - Have patient breathe deeply and cough several times to receive deep specimen.
    - Patient should expectorate into dry, sterile container.
- If patient is unable to produce sputum, induce using saline nebulization. Consult respiratory therapy for assistance.
- Transport immediately at ambient temperature. Refrigerate if a delay of more than one hour is anticipated; freeze for molecular tests.

Stool, Feces

- Collect specimen in a clean bed pan or use plastic wrap placed between the toilet seat and the bowl. Do not submit feces contaminated with urine or toilet water.
- Immediately transfer specimen into a clean, dry container or the appropriate preservative.*
- Transport unpreserved stool refrigerated or frozen.* *(Refer to chart on page 31)

Throat

- Use a cotton or Dacron swab.
- Use a tongue blade and an adequate light source to ensure proper visualization.
- Reach behind the uvula and swab:
    - both tonsillar fauces, and
    - the posterior pharynx, and
    - any ulceration, exudate, lesion or area of inflammation.
- Place the swab into the transport media and transport at ambient temperature or 2-8°C for viral cultures.

Urine

- Instructions for female patients to collect midstream urine for bacterial culture
  - Remove undergarments.
  - Wash hands thoroughly with soap and water, rinse them, and dry them on a disposable paper towel or shake off excess water.
  - Spread labia, with one hand, and keep them continuously apart.
  - Take the open sterile cup in the other hand without touching the rim or inner surface of the cup or lid.
  - Void 20 to 25 mL into the toilet and catch a portion of the rest of the urine in the container without stopping the stream. Do not touch the legs, vulva, or clothing with the cup.
  - Place the lid on the cup.
- Instructions for male patients to collect midstream urine for bacterial culture
  - Wash hands.
  - Retract the foreskin completely.
  - Void 20 to 25 mL into the toilet and catch a portion of the remaining urine in the cup without stopping the stream. Do not touch the cup with the penis.
  - Place the lid on the cup.
- Specimen handling
  - Label the container immediately and refrigerate at 2-8°C within 10 minutes of collection or transfer > 2 mL urine into a boric acid transport tube.

Wounds (open)

- Clean the sinus tract opening of the wound surface mechanically, without using a germicidal agent, to remove as much of the superficial flora as possible.
- Attempt to culture the base or edges of the wound to avoid collecting "normal flora" organisms.
- The following are preferred specimens for sinus tracts
  - Aspiration material obtained by needle or catheterization.
  - Curettings from the lining of the sinus tract.
- Specimen swabbings of sinus tracts are acceptable only if the above cannot be obtained. Swabs of sinus tracts may not accurately reflect underlying disease process.
- Do not submit cultures of superficial lesions for anaerobic culture. Biopsy of advancing margin of wound is the preferred specimen for anaerobes, mycobacteria, and fungi.

The Normal Flora

In a healthy animal, the internal tissues, e.g. blood, brain, muscle, etc., are normally free of microorganisms. On the other hand, the surface tissues, e.g. skin and mucous membranes, are constantly in contact with environmental organisms and become readily colonized by certain microbial species. The mixture of organisms regularly found at any anatomical site is referred to as the normal flora. The normal flora of humans is exceedingly complex and consists of more than 200 species of bacteria. The makeup of the normal flora depends upon various factors, including genetics, age, sex, stress, nutrition and diet of the individual. The normal flora of humans consists of a few eukaryotic fungi and protists, and some methanogenic Archaea that colonize the lower intestinal tract, but the Bacteria are the most numerous and obvious microbial components of the normal flora. The distribution of the bacterial flora of humans is

shown in Table 1. This table lists only a fraction of the total bacterial species that occur as normal flora of humans, and it does not express the total number or concentration of bacteria at any site.

TABLE 1. BACTERIA COMMONLY FOUND ON THE SURFACES OF THE HUMAN BODY

BACTERIUM	Skin	Conjunctiva	Nose	Pharynx	Mouth	Lower Intestine	Anterior urethra	Vagina
Staphylococcus epidermidis	++	+	++	++	++	+	++	++
Staphylococcus aureus*	+	+/-	+	+	+	++	+/-	+
Streptococcus mitis				+	++	+/-	+	+
Streptococcus salivarius				++	++			
Streptococcus mutans*				+	++			
Enterococcus faecalis*				+/-	+	++	+	+
Streptococcus pneumoniae*		+/-	+/-	+	+			+/-
Streptococcus pyogenes*	+/-	+/-		+	+	+/-		+/-
Neisseria sp.		+	+	++	+		+	+
Neisseria meningitidis*			+	++	+			+
Veillonellae sp.					+	+/-		
Enterobacteriaceae* (Escherichia coli)		+/-	+/-	+/-	+	++	+	+
Proteus sp.		+/-	+	+	+	+	+	+
Pseudomonas aeruginosa*				+/-	+/-	+	+/-	
Haemophilus influenzae*		+/-	+	+	+			
Bacteroides sp.*						++	+	+/-
Bifidobacterium bifidum						++		
Lactobacillus sp.)				+	++	++		++
Clostridium sp.*					+/-	++		
Clostridium tetani						+/-		
Corynebacteria	++	+	++	+	+	+	+	+
Mycobacteria	+		+/-	+/-		+	+	
Actinomycetes				+	+			
Spirochetes				+	++	++		
Mycoplasmas				+	+	+	+/-	+

++ = nearly 100 percent   + = common   +/- = rare   * = potential pathogen

Very little is known about the nature of the associations between humans and their normal flora, but they are thought to be dynamic interactions rather than associations of mutual indifference. Both host and bacteria are thought to derive benefit from each other, and the associations are, for the most part, mutualistic. The normal flora derives from the host a supply of nutrients, a stable environment and constant temperature, protection, and transport. The host obtains from the normal flora certain nutritional benefits, stimulation of the immune system, and colonization strategies that exclude potential pathogens at the site.

The normal flora are obviously adapted to their host (tissues), most probably by biochemical interactions between bacterial surface components (ligands or adhesins) and host cell molecular receptors. A great deal of information is available on the nature of adhesion of bacterial pathogens to animal cells and tissues, and reasonably similar mechanisms should apply to the normal flora.

In general, there are three explanations for why the normal bacterial flora are located at particular anatomical sites.

1. The normal flora exhibit a tissue preference or predilection for colonization. Certain species of bacteria are invariably in one locale and never in another (See Table 1 above). This is sometimes referred to as tissue tropism (See Table 2 below). One explanation for tissue tropism is that the host provides an essential growth factor needed by the bacterium. Of course, to explain why bacteria are

not at an alternative site, the host inherently provides an inhospitable environment for the bacterium by the production of such substances as stomach acids, bile salts and lysozyme.

2. Many, perhaps most, of the normal flora are able to specifically colonize a particular tissue or surface using their own surface components (e.g. capsules, fimbriae, cell wall components, etc.) as specific ligands for attachment to specific receptors located at the colonization site (See Table 3)

3. Some of the indigenous bacteria are able to construct bacterial biofilms on a tissue surface, or they are able to colonize a biofilm built by another bacterial species. Many biofilms are a mixture of microbes, although one member is responsible for maintaining the biofilm and may predominate.

TABLE 2. EXAMPLES OF TISSUE TROPISM OF SOME BACTERIA ASSOCIATED WITH HUMANS

BACTERIUM	TISSUE
Corynebacterium diphtheriae	Throat
Neisseria gonorrhoeae	Urogenital epithelium
Streptococcus mutans	Tooth surfaces
Streptococcus salivarius	Tongue surfaces
Vibrio cholerae	Small intestine epithelium
Escherichia coli	Small intestine epithelium
Staphylococcus aureus	Nasal membranes
Staphylococcus epidermidis	Skin

TABLE 3. EXAMPLES OF SPECIFIC ATTACHMENTS OF BACTERIA TO HOST CELL OR TISSUE SURFACES

Bacterium	Bacterial ligand for attachment	Host cell or tissue receptor	Attachment site
Streptococcus pyogenes	Protein F	Amino terminus of fibronectin	Pharyngeal epithelium
Streptococcus mutans	Glycosyl transferase	Salivary glycoprotein	Pellicle of tooth
Streptococcus salivarius	Lipoteichoic acid	Unknown	Buccal epithelium of tongue
Streptococcus pneumoniae	Cell-bound protein	N-acetylhexosamine-galactose disaccharide	Mucosal epithelium
Staphylococcus aureus	Cell-bound protein	Amino terminus of fibronectin	Mucosal epithelium
Neisseria gonorrhoeae	N-methylphenyl- alanine pili	Glucosamine-galactose carbohydrate	Urethral/cervical epithelium
Enterotoxigenic E. coli	Type-1 fimbriae	Species-specific carbohydrate(s) (e.g. mannose)	Intestinal epithelium
Uropathogenic E. coli	Type 1 fimbriae	Complex carbohydrate	Urethral epithelium
Uropathogenic E. coli	P-pili (pap)	Globobiose linked to ceramide lipid	Upper urinary tract
Bordetella pertussis	Fimbriae ("filamentous hemagglutinin")	Galactose on sulfated glycolipids	Respiratory epithelium
Vibrio cholerae	N-methylphenylalanine pili	Fucose and mannose carbohydrate	Intestinal epithelium
Treponema pallidum	Peptide in outer membrane	Surface protein (fibronectin)	Mucosal epithelium
Mycoplasma	Membrane protein	Sialic acid	Respiratory epithelium
Chlamydia	Unknown	Sialic acid	Conjunctival or urethral epithelium

TABLE 4. FREQUENTLY ENCOUNTERED BACTERIA IN PLAQUE, DENTAL CARIES, GINGIVITIS AND PERIODONTITIS

BACTERIUM	Plaque	Dental caries	Gingivitis	Periodontitis
Streptococcus sanguis	++	++	++	+
S. mutans	++	++	0	0
S. salivarius	0	0	0	0
Actinomyces viscosis	+	+	++	+
A. israelii	+	+	++	++
Lactobacillus sp.	+	+	0	0
Propionibacterium acnes	0	+	+	++
Bacteroides sp.	0	0	+	++
Selenomonas sputagena	0	0	+	++
Large spirochetes	0	0	0	++

++ = Frequently encountered in high proportions; + = Frequently encountered in low to moderate proportions; 0 = Sometimes encountered in low proportions or not detectable.

Normal Flora of the Gastrointestinal Tract.

The bacterial flora of the GI tract of animals has been studied more extensively than that of any other site. The composition differs between various animal species, and within an animal species. In humans, there are differences in the composition of the flora which are influenced by age, diet, cultural conditions, and the use of antibiotics. The latter greatly perturbs the composition of the intestinal flora. The following table shows the distribution of some common intestinal bacteria in various animal species including humans.

TABLE 5. NUMBERS OF VIABLE BACTERIA FOUND IN THE FECES OF ADULT ANIMALS (Log # viable cells per gram feces) *

Animal	E. coli	C. perfringens	Enterococci	Bacteroides	Lactobacilli
Cattle	4.3	2.3	5.3	0	2.4
Sheep	6.5	4.3	6.1	0	3.9
Horses	4.1	0	6.8	0	7.0
Pigs	6.5	3.6	6.4	5.7	8.4
Chickens	6.6	2.4	7.5	0	8.5
Rabbits	2.7	0	4.3	8.6	0
Dogs	7.5	8.4	7.6	8.7	4.6
Cats	7.6	7.4	8.3	8.9	8.8
Mice	6.8	0	7.9	8.9	9.1
Humans	6.7	3.2	5.2	9.7	8.8

* Median values from 10 animals

TABLE 6. BACTERIA FOUND IN THE LARGE INTESTINE OF HUMANS

BACTERIUM	Range of Incidence
Bacteroides fragilis	100
Bacteroides melaninogenicus	100
Bacteroides oralis	100
Lactobacillus	20-60
Clostridium perfringens	25-35
Clostridium septicum	5-25
Clostridium tetani	1-35
Bifidobacterium bifidum	30-70
Staphylococcus aureus	30-50
Enterococcus faecalis	100
Escherichia coli	100
Salmonella enteritidis	3-7
Salmonella typhi	0.00001
Klebsiella sp.	40-80
Enterobacter sp.	40-80
Proteus mirabilis	5-55
Pseudomonas aeruginosa	3-11
Peptostreptococcus sp.	common
Peptococcus sp.	moderate
Methanogens (Archaea)	common

ANTIBIOTIC ASSAY

The Agar-Diffusion Cylinder Cup Method

Cylinder cups – These are made of glass , porcelain or stainless steel,are 10 mm long , 6 mm inside diameter and open at both ends . They are known as heathley cups. The " fish-spine " insulators used to insulate electrical wiring exposed to heat are also used .
Petri dishes – to accommodate the cups on a deep layer of agar this must be 20 mm deep . Before sterilizing fit the lids (glass or aluminum) with circles of thick blotting paper to absorb water of syneresis during the incubation period .
Medium- use commercial (Difco or Oxoid) media designed for assay work for reproducible results . Two media are required one for the base layer tubed in 21 ml amounts , the other for the seed layer , tubed in 4 ml amounts.
Bacterial suspension – The organisms used for assay of different antibiotics are maintained on slopes of the seed medium. To prepare suspension for use wash the organisms from a 24 h slope culture with 2-3 ml of sterile saline and use all of this to inoculate a large slope in a 150 ml medical flat screw – capped bottle incubate for 24 h, wash off and suspend in 50ml saline Make serial ten fold dilutions of some of this suspension in saline and add 0.1ml amounts to 4 ml of melted seed medium at 45-50 c. pour on plates of base medium and allow to set . On each plate, place a Heatley cup and fill it with a solution of penicillin I ug per ml. Incubate and note plate which gives the most clear cut zone This is the dilution of suspension to be used in the test . Note turbidity of this dilution with a spectrophotometer or brown's opacity tubes and use it for future tests Standard suspensions will keep for several weeks in a refrigerator .
Standard dilutions of antibiotic – Make four doubling dilutions of the antibiotic in saline to include the MIC.
Test dilutions: Use serum 1:4 and 1:8 Exceptionally higher dilutions may be necessary. Dilute urine 1: 50, 1: 100 and 1: 200. Template for placing cups and measuring zones – on a piece of board or stiff card draw circles of 90 mm and 50mm diameter with a common centre.Draw two diameters at right angles. Calibrate each radius in millimeters with the zeros at the point of intersection of the diameters and inner circle .

Method

1-pour four plates each with 21ml base medium.seed 4 tubes of 4ml of melted medium at 45-50C each with 0.1 ml of bacterial suspensions and pour evenly on base medium.
2-Use template to position four Heatley cups on each plate , at intersections of radii and inner circle.
3-On two (reference) plates fill the cups with the four standard solutions of antibiotic.
4-On the other two (test) plates, fill three cups with the three test dilutions and one with the second or third of the standard dilutions. These act as controls on the test plates .
5-Incubate for exactly 24 h and measure the diameters of the zones of inhibition on the template or with a rule or dividers . An instrument for projecting a picture of the plate on a screen with a graduated scale is not difficult to devise .
6- Draw a graph of the diameters of the zones on the reference plates against the concentrations of antibiotic in the cups . Use this to determine the concentrations of the unknown . The reference dilution on the test plate should of course give the correct figure . If these tests are done daily and the reference graphs are consistent. They need not be included for each assay.the single refrence standard is sufficient .

ANTIBIOTIC SENSITIVITY TESTING

The disc-diffusion Method

This is the most commonly used method for investigating pyogenic and other acute infections. Several firms (Oxoid , and Difco) market blotting paper discs impregnated with antibiotics . Some drug companies give these to encourage the use of their products. Many laboratory workers prefer to make their own discs. Ford's blotting papers are available in a number of different colours and shades which assists in identilfying discs. Cut the discs5-6 mm in diameter with hand punch , sterilize in the hot-air oven in Petri dishes and either dip in a solution of antibiotic or spread out and give each disc 1 drop from a 50- dropper (0.02 ml).Dry the discs , still spread in Petri dishes in a vacuum dessicator over phosphorus pentoxide and store in screw – capped bottles over silica gel with indicator. Slow drying in an incubator and damp storage inactivates some antibiotics.

Method

Take a representative sweep or 3-4 colonies of the organism to be tested and suspend in 5 ml of nutrient broth There should be a faint opacity . pipette 2 or 3 ml on the surface of a lysed blood agar , allow to cover the suface, tilt and pipette off the surplus. (This is called making a lawn). Allow the plate to dry for 30 min in the incubator . Lawns can also be made from slightly denser suspensions with some practice by using a glass spreader . place the discs on medium using not more than four per plate , invert and incubate. Each day test the current discs against a culture of a known sensitive organism.

DISINFECTANTS AND ANTISPECTICS

THE LABORATORY TESTING OF DISINFECTANTS

The following are the main tests that have been devised for assessing the efficiency of disinfectants:

- **a)** The Minimal Inhibitory Concentration Test.
- **b)** The Rideal-Walker Test
- **c)** The Chick-Martin Test
- **d)** Capacity Use- Dilution Test
- **e)** The Stability Test
- **f)** The ‹In-Use' Test

A) THE MINIMAL INHIBITORY CONCENTRATION (MIC):

The Minimal Inhibitory Concentration (MIC) is the lowest concentration of disinfectant which will inhibit the growth of a standard culture of bacteria. It is assessed against the standard cultures of *S. typhi* to select the working dilutions of phenolic disinfectants to be used in the Rideal-Walker and Chick-Martin tests, and against the standard *E. coli, Pr. vulgaris, Staph. aureus* and *Ps. aeruginosa* cultures to select the most resistant organism to be used for the Capacity Use-Dilution test for both phenolic and non-phenolic disinfectants.

Method

Prepare 10 doubling dilutions of the disinfectant in nutrient broth as follows:

1. In a row of 10 sterile test tubes add 9.5 ml of nutrient broth to the 1st tube and 5 ml of the broth to the other 9 tubes.
2. Add 0.5 ml of the disinfectant to the 1st tube containing 9.5 ml of broth, thus making a 1 in 20 dilution, and mix well.

3. Withdraw 5 ml of the mixture from the first tube and add it to the second, mix well and then transfer 5 ml from the second to the third tube, and so on discarding 5 ml from the tenth tube. Use a separate pipette for each operation.
4. Inoculate each tube with 0.02 ml of one of the cultures with a '50' dropper pipette.
5. Incubate each tube at 32C and examine for growth after 72 h.
6. Verify the results by subculture to nutrient agar.

The Minimal Inhibitory Concentration is the highest dilution which fails to show growth.

B) THE RIDEAL-WALKER TEST

1. Find the MIC of the disinfectant.
2. Make five dilutions of the disinfectant, differing by 1 in 100, so that the second dilution is the MIC (e.g. if the MIC is 1 in 500 let the dilutions be 1 in 400 ; 1 in 500 ; 1 in 600; 1 in 700 and 1 in 800).
3. Make a 5 percent solution of phenol (BP or USP m.p. 40.5C) and prepare five dilutions 1 in 95; 1 in 100;1in 105; 1 in 110 and 1 in 115.
4. Set up 4 rows each of 5 tubes of nutrient broth.
5. In front of these set out 5 ml amounts of the 5 dilutions of disinfectant. The tubes should be kept in a water bath at 18C.
6. At 30 s intervals add 0.2 ml of a 24 h culture of *S. typhi* (NCTC 786) to each of the dilutions commencing with the lowest dilution. This takes 2 min.
7. After 30 s remove 1 loopful (with a 28 gauge write having a 4mm internal diameter loap) from the first dilution tube to the first tube in the front row of broth tubes.
8. Thirty seconds later inoculating the tubes in the front row of broth tubes.
9. Continue inoculating the tubes in front row at 30 s intervals. Thus each broth tube in this row recieves its inoculum 2.5 min after the organisms were added to the disinfectant dilutions.
10. Repeat this operation again with the second row of broth tubes at 30 s intervals so that each tube here receives its inoculum 5 min after the organisms were added to the disinfectant dilutions.
11. Repeat with the third row of broth tubes after 7.5 min exposure of the organisms to the disinfectant.
12. Repeat with the fourth row of broth tubes after 10 Min exposure.
13. Duplicate the procedure with the phenol dilutions.
14. Incubate the broths for 48 h at 37C.

To calculate the phenol coefficient let (a) be the dilution of disinfectant which allows growth at 2.5 min and 5 min but not at 7.5 min and (b) the dilution of phenol which allows growth at 2.5 min and 5 min but not at 7.5 min.

$$\text{The Phenol Coefficient} = \frac{(a)}{(b)}$$

For greater accuracy the test should be done in duplicate.

For the full details of the technique, see also 'Technique for Determining the Rideal-Walker Coefficient of Disinfectants', British Standard Specification No.5419 .

C)THE CHICK-MARTIN TEST FOR DISINFECTANTS

In the test as originally described, dried faces were added to the disinfectant as it was required to act in the presence of organic material, which diminishes the activity of many disinfectants. There are objections to the use of faeces and Garrod (British Standards Specification No.808)10 , modified the test by substituting a standardized yeast suspension for dried faeces. The following is an outline of the test procedure:

1. Find the MIC of the disinfectant and make up a 1,2 or 5 percent solution, whichever is nearest to twice the MIC.
2. Prepare a 5 percent solution of pure phenol (B.P. or U.S.P. m.p. 40.5C).

3. Make up a 5 per cent suspension of commercial dried yeast in distilled water and autoclave it.
4. Set up a row of 9 test-tubes, to the first tube and add 2.5ml amounts of dilutions, falling by 10 per cent, into the remaining 8 tubes.
5. Set up another row of 9 test-tubes and add 2.5ml of the 5 per cent phenol solution and 2.5ml amounts of dilutions, falling by 10 per cent, into the remaining 8 tubes.
6. To 48ml of the autoclaved yeast suspension add 2ml of an overnight broth culture of *S. typhi* (NCTC 3390) and mix.
7. Set up rows of 10ml nutrient broth, one for each dilution of the disinfectant under test and of the phenol.
8. At 30 s intervals pipette 2.5ml of the yeast- S.typhi mixture to the successive tubes of disinfectant dilutions to successive tubes of phenol dilutions. Leave on the bench at room temperature.
9. Exactly 30 min after the first dilution tube received its yeast and bacterium mixture, remove one loopful (with a 28 s.w.g. 4 mm i.d. loop bent at right angles) from this tube to a corresponding tube of nutrient broth.
10. At 30s intervals transfer loopfuls from the other dilution tube in succession to corresponding tubes of broth.
11. Incubate the broth tubes for 24 h and note the dilution of the tube which fails to show growth and the first tube which shows growth in both the disinfectant and phenol series. Determine the Phenol Coefficient by dividing the mean of those concentrations of Phenol which do and do not show growth with the mean of those concentrations of disinfectant which do not show growth.

Example.

Phenol : No growth in 1.62 per cent dilution
but growth in 1.46 per cent
mean = 1.54

Disinfectant : No growth 0.45 per cent dilution
but growth in .41 per cent
mean = 0.43

$$\text{Phenol Coefficient} = \frac{1.54}{0.43}$$

VIABLE COUNTS

In these techniques the material containing the bacteria is serially diluted and aliquots of each dilution placed in or on suitable culture media. Each colony developing is assumed to have grown from one viable unit, , may be one organism or a group of many.

Diluents Some diluents, e.g. saline or distilled water, may be lethal for organisms. Diluents must not be used direct from the refrigerator as cold-shock may prevent organisms from reproducing. Ringer solution is a better diluent than saline but 0.1 percent peptone water is probably the safest.

Preparation of Dilutions

1-Pipettes used in making dilutions must be very clean, otherwise bacteria will adhere to their inner surfaces and may be washed out into another dilution. Siliconed pipettes may be used. Fast-running pipettes and vigorous blowing-out should be avoided. As much as 0.1 ml may remain in pipettes if they are improperly used.

2-Pipette 9 ml amounts of diluent solution into sterile test-tubes with aluminium caps. These are the dilution blanks. When diluting liquids, for example, milk for bacterial counts, proceed as follows:
3- Mix sample by shaking. With a straight side pipette dipped 25mm into liquid, remove 1 ml. Deliver into the first dilution blank, about 25mm above the level of the liquid. Wait 3 s and blow out, discard pipette.
4- With a fresh pipette dip about 25 mm into liquid, suck up and down ten times to mix, but do not blow bubbles. Raise pipette and blow out. Remove 1 ml and transfer to next dilution blank. Discard pipette. Continue for the required number of dilutions, and remember to discard pipette after delivering contents, otherwise the liquid on the outside will contribute to a cumulative error. The dilutions will be :

Tube No.	1	2	3	4	5
Dilution	1/10	1/100	1/1000	1/10000	1/1000000
Vol. of original	0. 1	0.01	0.001	0.0001	0.00001 ml
Fluid per ml or	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5} ml

5-When counting bacteria in solid or semisolid material weigh out 10 g and place in Atomix or Waring Blender. Add 90 ml diluent, cover and homogenize.
6-Alternatively, cut into small pieces with a sterile scalpel, mix 10 g with 90 ml of the diluent and shake well. Allow to settle. Assume that bacteria are now evenly distributed between solid and liquid. Both of these represent dilutions of 1/10, i.e. 1 ml contains or represents 0.1 g and further dilutions are prepared as above. The dilutions will be:

Tube No.	1	2	3	4
Dilution	1/100	1/1000	1/10000	1/100000
Weight of original	0.01	0.001	0.0001	0.00001 g
Material	10^{-2}	10^{-3}	10^{-4}	10^{-5} g

1- Pour Plate Count

Melt nutrient agar or other suitable media tubed in 10 ml amounts. Cool to 45C in water bath. Set out Petri dishes, two or more per dilution to be tested. Label with dilution number. Pipette 1 ml of each dilution into the center of the appropriate dishes. Use a fresh pipette for each dilution. Do not leave the dish uncovered for longer than is absolutely necessary. Add content of one agar tube to each dish in turn and mix as follows : Move dish gently six times in a clockwise circle, diameter about 150 mm. Repeat with to-and-fro movement. Allow medium to set, invert and incubate at 37C for 24-48h.

To count, select plates showing between 30 and 300 colonies. Use a colony counting box place the open dish glass side up and if necessary, divide into sectors by ruling through the diameter with pen and ink. There may be too many colonies to count the whole plate. Count, using a 75mm magnifier and mark the glass above each colony with ink. Calculate colony count by multiplying the average number of colonies counted per countable plate by the reciprocal of the dilution and report as ‹ colony count per gram or milliliter.' Astell, Denley and Gallenkamp supply colony counting boxes. If large numbers of plates have to be counted, it is worth purchasing one of the more sophisticated Gallenkamp or Scientifica models. Instead of counting or using a tally counter, touching the plate with the probe records on a transistorized digital counter.

2-Roll Tube Count

Instead of using plates , tubes or bottles containing media are inoculated with diluted material and rotated horizontally until the medium sets .After incubation, colonies are counted . Tube medium in 2-4 ml amounts in screw -capped bottles. The medium should contain 0.5 to 1.0 per cent more agar than is usual . Melt and cool to 45 C in water bath. Add 0.1 ml of each dilution and rotate horizontally in cold water until agar is set in a uniform film around the inner walls of the bottle. This requires some practice . An alternative method employs a slab of ice taken from the ice tray of a refrigerator . Turn it upside down on a

cloth and make groove in it by rotating horizontally on it a bottle similar to that used for the counts but contatining warm water The count tubes are then rolled in this groove . if the roll- tube method is to be used often , an Astell-type roll- tube water bath saves much time and labour. Incubate roll tube cultures inverted so that condensation water collects in the neck and does not smear colonies growing on the agar surface. To count, draw a line parallel to the long axis of the bottle and rotate the bottle counting colonies under a low power magnifier .

3 -Drop Count Method

In this method , introduced by Miles and Misra and usually referred to by their names , small drops of the material are placed on agar plates . Colonies are counted in the inoculated area after incubation .

Prepare 50- Doppers: These are Pasteur pipettes passed through a starratt Standard Wire Gauge hole No .59 (0.95 mm) and cut off accuratcly above the hole When tested they should deliver 0.02 ml i.e. 50 drops/ml a tolerance of + or -2 drops is permissible . alternatively and preferably , use hypodermic needles 19 gauge before the bevels and points are ground . Clean well with chloroform to remove grease before testing as it affects accuracy . Attach to a short piece of glass tubing by a rubber sleeve use with a rubber teat . when tested , about 70-80 percent of these deliver between 48 and 52 drops /ml. Clean 50droppers of either kind in hot detergent , rinse in chloroform and sterilize in glass tubes .

Dry plates of suitable medium very well before use . Drop at least 5 drops from each dilution of sample from a height of not more 10 mm (to avoid splashing) on each plate. Replace lid but do not invert until drops have dried . After incubation, select plates showing discrete colonies in each drop using hand magnifier . Divide total count by number of drops counted , multiply by 50 to convert to 1 ml and by dilution used .

This method lends itself to arbitrary standards ; for example , if there are less than 10 colonies between 5 drops of sample this represents less than 100 colonies / ml of that material if these count per millilitere is greater than 2000

4-Surface Count Method

This is not very accurate but is useful for rough estimates of bacterial numbers e.g in urine exanination or 'inline 'checks in food processing plant . Place 0.1 or other suitable amount of the sample measured with a standard loop or a microsyringe or heamoglobin pipette in the centre of a well – dried plate of suitable medium and spread it with a loop or spreader all over the surface, incubate and count colonies. When counting colonies difficulties arise with spreading organisms and large " smears" of small colonies caused when a large viable unit. Uusally other clonies can be seen and counted through spreaders.

DNA Fingerprintion

Background

Restriction enzymes recognize specific short nucleotide sequences in double-stranded DNA and cleave both strands of the molecule (Table 30.1). Restriction enzymes are bacterial enzymes that restrict the host range of bacteriophages. The enzymes were discovered in laboratory experiments when phages were used to infect bacteria other than their usual hosts. Restriction enzymes in the new host destroyed the phage DNA. Today, over 300 restriction enzymes have been isolated and purified for use in DNA research. Each enzyme recognizes a different nucleotide sequence in DNA. The enzymes are named with three-letter abbreviations for the bacteria from which they were isolated.

Recognition Sequences of Some Restriction Endonucleases

Enzyhme	Bacterial Source	Recognition Sequence
EcoR I	*Escherichia coli*	G↓AATTC CTTAA↑G
Hae III	*Haemophilus aegyptius*	GG↓CC CC↑GG

DNA cleaved with restriction enzymes produces restriction fragment length polymorphisms (RFLPs). The size and number of the pieces is determined by agarose gel electrophoresis, the digested DNA is placed near one end of a thin slab of agarose and immersed in a buffer to allow current to flow through the agaros. Electrodes are attached to both ends of the gel, and a current is applied. Each piece of DNA then migrates toward the positive electrode at a rate determined by its size. The DNA fragments are visualized by staining with methylene blue or ethidium bromide.

These enzymes can be used to characterize DNA because a specific restriction enzyme will cut a molecule of DNA everywhere a specific base sequence occurs. When the DNA molecules from two different microor-ganisms are treated with the same restriction enzyme, the restriction fragments that are produced can be separated by electrophoresis. A comparison of the number and sizes of restriction fragments produced from different organisms provides information about their genetic similarities and differences, the more similar the patterns, the more closely related the organisms are expected to be.

In forensic science, analyses of DNA can be used to determine the father of a child or the perpetrator of a crime. DNA samples are digested to produce RFLPs that are separated by gel electrophoresis. The DNA fragments are transferred to a membrane filter by blotting so the fragments on the paper are in the same positions as the fragments on the gel. The paper is then heated to produce single strands of DNA. Small pieces of radioactively labeled single-stranded DNA called probes are used to distinguish core sequences of nucleotides. When the probe is added to the gel, it will anneal to its complementary strand. The gel can then be placed on photographic film, and the radioactive label will expose the film to produce an autoradiograph.

In this exercise, we will compare plasmids from an unknown bacterial species to plasmids from known bacteria in order to identity the unknown.

Materials

Digestion of DNA

DNA samples, Restriction enzyme, Restriction buffer, Micropipette, 1-10 μl, Micropipette tips (13), Microcentrifuge tubes (5).

Electrophoresis of DNA Samples

Electrophoreses buffer, Casting tray and comb, Agarose, 0.8 %, Tracking dye, Electrophoresis chamber and power supply, Ethidium bromide or methylene blue and Transilluminator or light box.

Procedure

Every pair of students will perform five digests: One each from the unknown and four known bacteria.

digestion of DNA

1. Label five microcentrifuge tubes "U," "1," "2," "3," and "4."

2. To each tube add 5 μ l restriction buffer. Using a new pipette tip, add 4μ l restriction enzyme to eac
tube.
3. add the DNA samples to each tube as follows. Use a different pipette tip for each sample. Why?

Tube	DNA
U	Unknown, 4 μ l
1	species, 1,4 μ l
2	species, 2,4 μ l
3	species, 3,4 μ l
4	species, 4,4 μ l

4. centrifuge the tubes for 1 to 2 seconds to mix. Be sure to balance the centrifuge. Then incubate th tubes in a water bath at 37C for 45 minutes. After incubation, the tubes can be frozen whe electrophoresis will be done during another lab period.

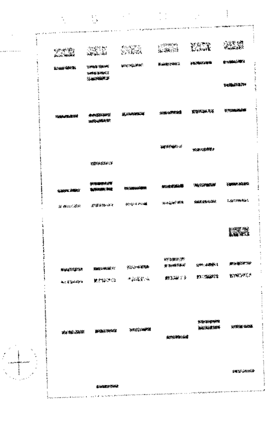
Electrophoresis of DNA Samples

1. Use the melted agarose to pour a gel.
2. If necessary, defrost the tubes of digested DNA by holding them in your hand or placing them in a 37C water bath.
3. Add 1 μ l of tracking dye to each tube. Centrifuge the tubes for 1 to 2 seconds to mix. *Be sure the centrifuge is balanced.*
4. load 14 μ l of one of your samples prepared above into a well. Using a different pipette tip, load you other samples.
5. Once the wells have been filled, apply power (125V) to the chamber. During the run, you will se the tracking dye migrate. The tracking dye will separate into its two component dyes durin electrophoresis. Turn off the power before the faster dye runs off the gel. Good separation of th DNA fragments occurs when the two dyes have separated by 4 or 5 cm. Run the gel until the dye i near the end of the gel, then turn the current off and remove the gel.
6. To stain the gel, use either step a or step b below.

a) Transfer the gel to the ethidium bromide staining tray for 5 to 10 minutes. Wear safety goggles. Do no touch the ethidium promide. It is a mutagen. Transfer the gel to tap water to destain for 5 minutes. (Chlorin in tap water will inactivate residual ethidium.) Place your gel on the transilluminator and close the plasti lid. Do not look directly at the UV light. Do not turn the UV light on until. the plastic lid is down. Ethidiu bromide that is bound to DNA did not wash off and will fluoresce with UV light.
b) Transfer the gel to the methylene blue staining tray for 30 minutes to 2 hours. Destain by placing th gel in water for 30 minutes to overnight. Place your gel on a light box Methylene blue that is bound to DNA did not wash off.

7. Carefully draw the location of the bands or photograph your gel using the transilluminator camera.

Exercise: The following agarose gel electrophoresis patterns were obtained from *Eco*R 1 digests of DNA from different isolates. The gels were developed with a DNA probe for a particular guanine-cytosine sequence. Which appear to be most closely related? Do any appear to be the same species? Briefly explain how you arrived at your conclusions.



Microbes in Water

Multiple-Tube Technique

Background

Tests that determine the bacteriological quality of water have been developed to prevent transmission of water-borne diseases of fecal origin. However, it is not practical to look for pathogens in water supplies because pathogens occur in such small numbers that they might be missed by sampling. Moreover, when pathogens are detected, it is usually too late to prevent occurrence of the disease. Rather, the presence of indicator organisms is used to detect fecal contamination of water. An indicator organism must be present in human feces in large numbers and must be easy to detect. The most frequently used indicator organisms are the coliform bacteria. Coliforms are aerobic or facultatively anaerobic, gram-negative, nonendospore-forming, rod-shaped bacteria that ferment lactose with acid and gas formation within 48 hours at 35C. Coliforms are not usually pathogenic, although they can cause opportunistic infections. Coliforms are not restricted to the human gastrointestinal tract but may be found in other animals and in the soil. Tests that determine the presence of fecal coliforms (of human origin) have been developed. Established public health standards specify the maximum number of colitorms allowable in each 100 ml. of water, depending on the intended use of the water (for example, drinking, water-contact sports, or treated wastewater for irrigation or for discharge into a bay or river).Coliforms can be detected and enumerated in the multiple-tube technique. In this method.

Analysis of drinking water for coliforms by the multiple-tube technique.

Most Probable Number (MPN) Index for Various Combinations of Positive and Negative Results When Three 10 m Portions. Three 1 ml portions, and Three 0.1 ml Portions re used.

Number of tubes giving positive reaction out of			MPN index per 100 ml	Number of tubes giving positive reaction out of			MPN index per 100 ml
3 of 10 ml Each	3 of 1 ml Each	3 of 0.1 ml Each		3 of 10 ml Each	3 of 1 ml Each	3 of 0.1 ml Each	
0	0	0	< 3	3	0	0	23
0	0	1	3	3	0	1	39
0	1	0	3	3	0	2	64
1	0	0	4	3	1	0	43
1	0	1	7	3	1	1	57
1	1	0	7	3	1	2	120
1	1	1	11	3	2	0	93
1	2	0	11	3	2	1	150
2	0	0	9	3	2	2	210
2	0	1	14	3	3	0	240
2	1	0	15	3	3	1	460
2	1	1	20	3	3	2	1.100
2	2	0	21	3	3	3	≥ 2.400
2	2	1	28				

Source: Standard Methods for the Examination of Water and Wastewater, 13th ed. New York;American Public Health Association, 1971

Coliforms are detected in two stages. In the presumptive test, dilutions from a water sample are added to lactose fermentation tubes. The lactose broth can be made selective for gram-negative bacteria by the addition of lauryl sulfate or brilliant green and bile. Fermentation of lactose to gas is a positive reaction.

Samples from the positive presumptive tube at the highest dilution are examined for coliforms by inoculating a differential medium in the confirmed test. A confirmed test can be done on 4-methylumbelliferone glucuronide (MUG) agar. Almost all strains of *E. Coli* produce the enzyme *B*-glucuronidase (GUD). If *E. coli* is added to a nutrient medium containing MUG. GUD converts MUG to a fluorescent compound that is visible with an ultraviolet lamp.

The number of coliforms is determined by a statistical estimation called the most probable number (MPN) method. In the presumptive test, tubes of lactose broth are inoculated with samples of the water being tested. A count of the number of tubes showing acid and gas is then taken, and the figure compared to statistical tables, shown in the above table. The number is the most probable number of coliforms per 100 ml of water.

Materials

Water sample, 50 ml (Bring your own, from a pond or stream.), 9 ml single-strength lactose fermentation tubes (6), 20 ml 1.5-strength lactose fermentation tubes (3), Sterile 10 ml pipette, Sterile 1 ml pipette and Petri plate containing MUG agar (second period).

Procedure

1-Label three single-strength lactose broth tubes "0.1" , label another three tubes "1", and label the three 1.5-strength broth tubes "10".
2-Inoculate each 0.1 tube with 0.1 ml of your water sample.
3-Inoculate each 1 tube with 1.0 ml of your water sample.
4-Inoculate each 10 tube with 10 ml of your water sample. Why is 1.5-strength lactose broth used for this step ?
5-Incubate the tubes for 24 to 48 hours at 35°C.

6-Record the results of your presumptive test (Figure 52.1). Which tube has the highest dilution of the water sample?. Determine the number of coliforms per 100 ml of the original sample using Table 52.1 If a tube has gas, streak the MUG agar with the positive broth. Incubate the plate, inverted, for 24 to 48 hours at 35°C.
7-examine the plate using an ultraviolet lamp. Record the results of your confirmed test. How can you tell whether coliform colonies are present?

Microbes Used in the Production of Foods

Background

Microbial fermentations are used to produce a wide variety of foods. Fermentation means different things to different people. In industrial usage, it is any large-scale microbial process occurring with or without air. To a biochemist, it is the group of metabolic processes that release energy from a sugar or other organic molecule, do not require oxygen or an electron transport system, and use an organic molecule as a final electron acceptor. In this exercise, we will examine a lactic acid fermentation used in the production of food.

In dairy fermentations, such as yogurt production, microorganisms use lactose and produce lactic acid without using oxygen. In nondairy fermentations, such as wine production, yeast use sucrose to produce ethyl alcohol and carbon dioxide under anaerobic conditions. If oxygen is available, the yeast will grow aerobically, liberating carbon dioxide and water as metabolic end-products.

Historically, nearly every population used milk that had been fermented by selected microbes. The acids and antibiotics produced during fermentation prevented the growth of spoilage bacteria. These "sour" milks have varied from country to country depending on the source of milk, conditions of culture, and microbial "starter" used. The bacteria yield lactic acid, and the yeast produce ethyl alcohol. Currently, two fermented cow milk products, buttermilk and yogurt, are widely used.

Buttermilk is the fluid left after cream is churned into butter. Today, buttermilk is actually prepared by souting true buttermilk or by adding bacteria to skim milk and then flavoring it with butterflake. *Lactococcus lactis* ferments the milk, producing lactic acid (sour); and neutral fermentation products (diacetyls) are produced by Leuconostoc. Yogurt originated in the Balkan countries, goat milk being the primary source. Yogurt is milk that has been concentrated by heating and then fermented at elevated temperatures. Streptococcus produces lactic acid, and Lactobacillus produces the flavors and aroma of yogurt.

Materials

Homogenized milk,Nonfat dry milk, Large sterile beaker, Sterilized stirring rod, Thermometer, Sterile glass test tube, Hot plate or ring stand and asbestos pad, Sterile 5 ml pipette and Styrofoam or paper cups with lids

Second period ; Plastic spoons, Petri plate containing trypticase soy agar, pH paper, gram stain reagents optional: jam, jelly, honey, and so on.

Culture

Commercial yogurt or *Streptococcus thermophilus* and *Lactobacillus bulgaricus*.

Procedure

Be sur e all glassware is clean.

1. Add 100 ml of milk per person (in your group) to a wet beaker (wash out the beaker first with water to decrease the sticking of the milk). Put a thermometer in a glass test tube containing water before placing it in the beaker.
2. heat the milk on a hot plate or over a burner on an asbestos pad placed on a ring stand, to about 85C for 10 to 20 minutes. Stir occasionally. Do not let it boil. Why is the milk heated?
3. Cover and cool the milk to about 65C. Add 3 g of nonfat dry milk per person. Stir to dissolve. Why is dry milk added?
4. Rapidly cool the milk to about 45C. Pour the milk equally into the cups.
5. Inoculate each cup with 1 to 2 teaspoonfuls of commercial yogurt, or 2.5 ml *S. thermophilus* and 2.5 ml *L. bulgaricus*. Cover and label the cups. Make a smear with the inoculum on a slide, heat-fix, and save for the second period.
6. incubate the cups at 45C for 4 to 18 hours or until they are firm (custardlike).
7. Cool the yogurt to about 5C. Save a small amount for steps 8 through 10. then taste it with a clean spoon. Add jam or some other flavor if you desire. Eat and enjoy !
8. Determine the pH of the yogurt.
9. Make a smear, next to the smear prepared previously, and after heat-fixing it, Gram stain it. Record your results.
10. Streak for isolation on trypticase soy agar. Incubate the plate, inverted, at 45C.

After distinct colonies are visible, record your observations.Prepare Gram stains from each different colony;

Preparation of heat killed bacterial vaccine

Vaccination is the administration of antigenic material to produce immunity to a disease. Effective vaccines change the immune system by promoting the development of antibodies (active immunization) that can quickly and effectively attack disease causing microorganisms or viruses when they enter the body preventing disease development. Vaccines are used as prophylactic (e.g. to prevent or ameliorate the effects of a future infection by any natural or "wild" pathogen). A vaccine administration may be oral, by injection (intramuscular, intradermal, subcutaneous), by puncture, transdermal or intranasal.

Vaccines may be dead or inactivated organisms or purified products derived from them. There are four types of traditional vaccines:

- **Vaccines containing killed microorganisms:** these are previously virulent microorganisms that have been killed with chemicals, radiation or heat. Examples are vaccines against flu, cholera, bubonic plague, and hepatitis A.
- **Vaccines containing live, attenuated microorganisms:** These are live micro-organisms that have been cultivated under conditions that disable their virulent properties or which use closely-related but less dangerous organisms to produce a broad immune response. They typically provoke more durable immunological responses and are the preferred type for healthy adults. Examples include yellow fever, measles, rubella, and mumps.
- **Toxoids:** These are inactivated toxic compounds in cases where these (rather than the micro-organism itself) cause illness. Examples of toxoid-based vaccines include tetanus and diphtheria.

- **Subunit:** Rather than introducing an inactivated or attenuated micro-organism to an immune system a fragment of it can create an immune response. Characteristic examples include the subunit vaccine against HBV that is composed of only the surface proteins of the virus (produced in yeast) and the virus-like particle (VLP) vaccine against human papillomavirus (HPV) that is composed of the viral major capsid protein.

Additives used in the production of vaccines may include:

- Suspending fluid (e.g. sterile water, saline, or fluids containing protein)
- Preservatives and stabilizers to help the vaccine remain unchanged (e.g. albumin, phenols, and glycine)
- Adjuvants or enhancers that help the vaccine improve its work.

Common substances found in vaccines include:

- **Aluminum** gels or salts of aluminum which are added as adjuvants to help the vaccine stimulate production of antibodies to fight off diseases and aid other substances in their action. In vaccines adjuvants may be added to help promote an earlier response, more potent response, or more persistent immune response to disease.
- **Antibiotics** which are added to vaccines to prevent the growth of germs (bacteria) in vaccine cultures.
- **Egg protein** which is found in vaccines prepared using chick embryos. Ordinarily, persons who are able to eat eggs or egg products safely can receive these vaccines.
- **Formaldehyde** which is used to inactivate bacterial products for toxoid vaccines, (these are vaccines that use a weakened or suppressed bacterial toxin to increase a response to an antigen or a disease such as the tetanus toxoid used to prepare tenanus vaccinations; toxoids are too weak or suppressed to be harmful). It is also used to kill unwanted viruses and bacteria that might be found in cultures used to produce vaccines.
- **Monosodium glutamate** (MSG) and 2-phenoxy-ethanol which are used as stabilizers in a few vaccines to help the vaccine remain unchanged even in the presence of forces such as heat, light acidity, humidity etc. MSG is also found in many foods, especially Asian foods and flavor enhancers.
- **Thimerosal** which is a preservative that might be added to prevent the vaccine from spoiling Thimerosal is also found in some contact lens solutions and throat sprays.

Antiserum is a serum containing antibodies that is obtained from an animal that has been exposed to antigen It is used in treatment of infectious disease by passing on passive immunity to many diseases.

Procedures:

An autogenous vaccine is one prepared from a patient's infecting strain, while a stock vaccine is prepared from standard strains.

Proceedure:

1-Seed pure culture of the organism heavily on nutrient agar or blood agar plates and incubate for 24 hours at 37°c.

2-Wash the growth off the surface of agar into saline, scraping of the medium very gently with a glass rod bent at right angle. It is important not to include particles of medium in the suspension.

3-The suspension is sterilized by minimal heating (heat at 60°c a water bath) as over heating may affect the antigenic properities of the dead organism.

4-Test for the sterility of the suspension by asceptic transfer of 1ml in to both fluid thioglycolate and fluid sabourand's medium.

5-Determine the total count of cells per ml in the suspension and adjust the concentration of the suspension by adding more saline untill the required strength is reached as judged by comparing the opacity with **Brown's** standard opacity tube.

6-Add chlorocresol or phenol to the sterile suspension as apreservative up to 0.1 or 5% concentrations respectively.

7-Transefer 20 ml of the suspension with strict aseptic precuations to a vaccine bottle. The bottles are closed aseptically and labelled in such a way so as to inicate:

For recurrent boils a vaccine containing 250 million *Staphylococci* per ml is suitable. A series of weekly injections is given starting with 0.1 ml and increasing by 1 each week for 10 weeks.
Streptococcus vaccines are prepared with less concentrated and contain about 50 million organism per m

Lable

a) Number of mls. In the bottle.
b) Name and concentration of the preservative.
c) Date of preparation.
d) Route of administration and dose.
e) Name of manufacturer
g) Storage and expiration.

STERILITY TESTING

The following procedures are applicable for determining whether a Pharmacopeial article purporting to be sterile complies with the requirements set forth in the individual monograph with respect to the test for sterility. Pharmacopeial articles are to be tested by the ***Membrane Filtration method*** under ***Test for Sterility*** of the Product to be Examined where the nature of the product permits. If the membrane filtration technique is unsuitable, use the ***Direct Inoculation of the Culture Medium*** method under ***Test for Sterility of the Product*** to he Examined. All devices, with the exception of Devices with Pathways Labeled Sterile, are tested using the **Direct** ***Inoculation of the Culture Medium*** method. Provisions for retesting are included under Observation and Interpretation of Results.
Because sterility testing is a very exacting procedure, where asepsis of the procedure must be ensured for a correct interpretation of results, it is important that personnel be properly trained and qualified. The test for sterility is carried out under aseptic conditions. In order to achieve such conditions, the test environment has to be adapted to the way in which the sterility test is performed. The precautions taken to avoid contamination are such that they do not affect any microorganisms that are to be revealed in the test. The working conditions in which the tests are performed are monitored regularly by appropriate sampling of the working area and by carrying out appropriate controls.
These Pharmacopeial procedures are not by themselves designed to ensure that a batch of product is sterile or has been sterilized. This is accomplished primarily by validation of the sterilization process or of the aseptic processing procedures.
When evidence of microbial contamination in the article is obtained by the appropriate Pharmacopeial method, the result so obtained is conclusive evidence of failure of the article to meet the requirements of the test for sterility, even if a different result is obtained by an alternative procedure.

MEDIA

Prepare media for the tests as described below, or dehydrated formulations may be used provided that, when reconstituted as directed by the manufacturer or distributor, they meet the requirements of the ***Growth Promotion Test of Aerobes, Anaerobes, and fungi.*** Media are sterilized using a validated process.
The following culture media have been found to he suitable for the test for sterility. ***Fluid Thioglycollate Medium*** is primarily intended for the culture of anaerobic bacteria. However, it will also detect aerobic bacteria. ***Soybean--Casein Digest Medium*** is suitable for the culture of both fungi and aerobic bacteria.

1-Fluid Thioglycollate Medium:

L.-Cystine	0.5 g
Sodium Chloride	2.5 g
Dextrose ($C_6H_{12}O_6 . H_2O$)	5.5/5.0 g
Agar, granulated (moisture content not exceeding 15%)	0.75 g
Yeast Extract (water-soluble)	5.0 g
Pancreatic Digest of Casein	15.0 g
Sodium Thioglyeollate	0.5 g
or Thioglycolic Acid	0.3 mL
Resazurin Sodium Solution (I in 1000), freshly prepared	1.0 ml.
Purified Water	1000 mL

Mix the L.-cystine, sodium chloride, dextrose, yeast extract, and pancreatic digest of casein with the purified water, and heat until solution is effected. Dissolve the sodium thioglycollate or thioglycolic acid in the solution and, if necessary, add 1 N sodium hydroxide so that, after sterilization, the solution will have a pH of 7.1 ± 0.2. If filtration is necessary, heat the solution again without boiling, and filter while hot through moistened filter paper. Add the resazurin sodium solution, mix, and place the medium in suitable vessels that provide a ratio of surface to depth of medium such that not more than the upper half of the medium has undergone a color change indicative of oxygen uptake at the end of the incubation period. Sterilize using a validated process. If the medium is stored, store at a temperature between 2° and 25° in a sterile, airtight container. If more than the upper one-third of the medium has acquired a pink color, the medium may be restored once by heating the containers in a water-bath or in free-flowing steam until the pink color disappears and by cooling quickly, taking care to prevent the introduction of nonsterile air into the container. N.B: Fluid Thioglycollate Medium is to be incubated at 32.5 ± 2.5°.

N.B:Alternative Thioglycollate Medium

Prepare a mixture having the same composition as that of the Fluid Thioglycollate Medium, **but omitting the agar** and the **resazurin sodium solution**, sterilize as directed above, and allow to cool prior to use. The pH after sterilization is 7.1 ± 0.2. Incubate under anaerobic conditions for the duration of the incubation period. Alternative Fluid Thioglycollate Medium is to be incubated at 32.5 ± 2.5°.

2-Soybean-Casein Digest Medium

Pancreatic Digest of Casein	17.0 g
Papaic Digest of Soybean Meal	3.0 g
Sodium Chloride	5.0 g
Dibasic Potassium Phosphate	2.5 g
Dextrose ($C_6H_{12}O_6.H_20$)	2.5/2.3 g
Purified Water	1000 ml

Dissolve the solids in the Purified water, heating slightly to effect a solution. Cool the solution to room temperature, and adjust the pH with I N sodium hydroxide so that, after sterilization, it will have a pH of 7.3

± 0.2. Filter, if necessary to clarify, dispense into suitable containers, and sterilize using a validated filtration process. Store at a temperature between 2° and 25° in a sterile well-closed container, unless it is intended for immediate use. Soybean-Casein Digest Medium is to be incubated at 22.5 ± 2.5 C.

Suitability Tests

The media used comply with the following tests, can be tested for its suitability before, or in parallel, with the test on the product to be examined.

Sterility

Confirm the sterility of each sterilized batch of medium by incubating a portion of the media at the specified inoculation temperature for 14 days. No growth of microorganisms occur.

Growth Promotion Test Of Aerobes, Anaerobes and Fungi

Test each lot of of ready-prepared medium for its growth promotion ability on the following suitable strains of microorganisms which are indicated in Table1. Inoculate portions of **Fluid Thioglycollate Medium** with number (not more than 100 cfu) of the following microorganisms using a separate portion of medium for each of the following of microorganism: *Clostridium sporogenes*, *Pseudomonus auriginosa*, and *Staphylococcus aureus*. Inoculate portions of. Fluid Thioglycollate Medium with a small number (not more than 100 cfu) of *Clostridium sporogenes*. Inoculate portions of **Soybean-Casein Digest Medium** with a small number (not more than 100 cfu of the following microorganisms, using a separate portion of the medium for each of the following species of microorganism: *Aspergillus niger*, *Bacillus subtilis*, and *Candida albicans*. Incubate for not more than 3 days in the case of bacteria and not more than 5 days in of fungi. The media are suitable if a clearly visible growth of microorganisms occurs.

Storage

If prepared media are stored in unsealed containers, they are used for 1 month, provided that they are tested for growth promotion within 2 weeks of the time of use. If stored in tight containers, the media used for 1 year, provided that they are tested for growth promotior within 3 months of the time of use.

Table I. Examples on Strains of the test microorganisms suitable for the growth promotion test and validation test:

Aerobic bacteria	
Staphylococcus aureus	ATCC 6538 NCTC 10788
Bacillus subtilis	ATCC 6633 NCIMB 80..
Pseudomonas aeruginosa	ATCC 9027 CIP 82.118
Anaerobic bacteria	
Closterdium sporogenes	ATCC 19404 NCTC 532
Fungi	
Candida albicans	ATCC 10231 NCPF 3179
Aspergillus niger	ATCC 16404

An alternative to *Staphylococcus aureus* is *Bacillus subtilis* 6633). An alternative to *Clostridium sporogenes* when a nonspore-forming microorganism is desired, is *Bacetroides vulgatus* (ATCC 8482). [NOTE-Seed-lot culture maintenance techniques (seed-lot system used so that the viable microorganisms used for inoculation

are not more than five passages removed from the original master seed lot.]In appropriate cases, periodic testing of the different batches prepared from the same lot of dehydrated medium is acceptable.

Direct Inoculation of the Culture Medium

Transfer the quantity of the preparation to be examined directly into the culture medium so that the volume of the product is not more than 10% of the volume of the medium, unless otherwise prescribed. If the product to be examined has antirnicrobial activity, carry out the test alter neutralizing this with a suitable neutralizing substance or by dilution in a sufficient quantity of culture Medium. When it is necessary to use a large volume of the product, it may be preferable to use a concentrated culture medium prepared in such a way that it takes into account the subsequent dilution. Where appropriate, the concentrated medium may be added directly to the product in its container.